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**Published paper**

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**In the absence of CD154, administration of IL-12 restores Th1 responses but not protective immunity to *Schistosoma mansoni*.<sup>1</sup>**

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<sup>3</sup> Abbreviations : DC, dendritic cell; p.c., post-challenge; p.v., post-vaccination; RA, radiation  
attenuated; sdLN, skin-draining lymph node; SSAP, soluble schistosomula antigen preparation.

## Abstract

The cytokine interplay during the development of protective immunity to the radiation-attenuated (RA) schistosome vaccine has been extensively characterised over recent years, yet the role of co-stimulatory molecules is much less well understood. Here we demonstrate for the first time the importance of CD40/CD154 interactions in vaccine induced immunity, as CD154<sup>-/-</sup> mice exposed to RA schistosomes develop no resistance to challenge infection. These mice have defective Th1-associated immune responses in the skin-draining lymph nodes and the lungs, with reduced or absent levels of IL-12p40, IFN $\gamma$ , and nitric oxide; the expression of MHCII on APCs recovered from the lungs of vaccinated CD154<sup>-/-</sup> mice also severely compromised. Anti-CD40 mAb did not reconstitute sustained Th1 responses in the lymph nodes, or the lungs, nor did the mAb restore anti-parasite IgG production, or protective immunity. On the other hand, administration of rIL-12 to CD154<sup>-/-</sup> mice shortly after vaccination caused elevated and sustained levels of Th1-associated cytokines, reconstituted the cellular composition and MHCII expression of CD11c<sup>+</sup> APCs from the lungs, and restored the appearance of inflammatory effector foci in the lungs. However, treatment of CD154<sup>-/-</sup> mice with rIL-12, did not restore protection. On the other hand, we were not able to demonstrate a role for antibody-mediated protective immunity since passive transfer of serum from vaccinated wild-type mice into vaccinated CD154<sup>-/-</sup> mice did not confer protection. We conclude that protective immunity to the RA schistosome vaccine is CD154-dependent but is independent of IL-12-orchestrated cellular immune mechanisms in the lungs.

## Introduction

Schistosomiasis is a parasitic infection of humans in many parts of the developing world, and it is estimated that 779 million people are at risk of infection, largely in sub-saharan Africa (1). Serious pathology results from granulomatous lesions that form in the liver, intestines, or bladder wall, which are the result of the host's immune response to ova produced by adult worms. Over recent decades, attention has focussed upon the development of a vaccine against schistosomiasis. The use of radiation-attenuated (RA) schistosome larvae has provided the most consistent way of inducing high levels of protection specifically against *Schistosoma mansoni* in a number of different mammalian hosts including experimental mice (2), and non-human primates (3).

Studies of RA vaccine-induced protective immunity in mice have revealed that resistance to challenge infection is dependent on CD4<sup>+</sup> Th1-type effector responses (4). For example, mice deficient in IL-12p40 (5, 6) or IFN $\gamma$ R (7) develop significantly reduced Th1-type responses during the induction and effector phases and exhibit low levels of protection. Recent studies have also identified the production of IL-12 by cells originating from the skin site of vaccination as being critical to the induction of biased Th1 immune responses (8, 9). Indeed, immune priming events in the skin and the balance of cytokine production, especially IL-12 *versus* IL-10, are important in determining the magnitude of the Th1 response in the skin-draining lymph nodes (sdLN) (9-11). In turn, immune events in the sdLN are an integral step in the ultimate priming of the lungs with IFN $\gamma$ -secreting CD4<sup>+</sup> cells (12), and the development of a cellular foci capable of eliminating challenge parasites (13).

Whilst the cytokine interplay during immune priming and the effector response after exposure to RA schistosome larvae has become clearer (2), the role of co-stimulatory molecules in the interaction between potential antigen presenting cells (APCs) and T lymphocytes is much less well understood. In this context, CD154 present on CD4<sup>+</sup> cells is a key molecule in the maturation of CD40-expressing APCs (14, 15). APCs activated via the CD154/CD40 pathway up-regulate other co-stimulatory molecules, such as CD80 and CD86, and inflammatory cytokines like IL-12 which together favour the development of Th1-type responses (16-20). Moreover, this pathway is critical for the development of protective Th1-mediated immunity to the intracellular parasites *Leishmania major* and *Toxoplasma gondii* (21, 22). We recently identified CD154 as an important molecule in the induction of Th1-associated cytokine production following exposure to RA larvae (23). In this situation, cells from the skin of mice deficient in CD154 failed to release optimum levels of IL-12p40, dermal APCs were less

mature, and CD4<sup>+</sup> cells from the sdLN of vaccinated mice failed to produce antigen-driven IFN $\gamma$ . However, we were able to recreate the Th1-biased immune response in vaccinated CD154<sup>-/-</sup> mice following administration of agonist anti-CD40 mAb or rIL-12. Similarly, anti-CD40 mAb boosts Th1 responses and inhibits Th2-type responses in mice immunised with schistosome eggs (24), while rIL-12 restores Th1 responses and protective immunity in CD154<sup>-/-</sup> mice exposed to *L. major* (21).

In the current study, we have defined the role of the CD154/CD40 co-stimulatory pathway in the development of immune effector responses in the lungs and the generation of protective immunity induced by RA schistosome larvae. In the absence of CD154, we show that protective immunity was totally absent and was associated with the abrogation of Th1 immune responses in the lungs. While agonist anti-CD40 mAb administered to groups of vaccinated CD154<sup>-/-</sup> mice only transiently restored Th1-mediated immune responses, administration of rIL-12 in the absence of CD154 had long term restorative effects. However, neither treatment was able to restore protective immunity. We conclude that endogenous CD154 is an obligatory component in the development of protective immune responses against schistosomes, but Th1-associated inflammatory foci in the lungs, induced following the administration of rIL-12, were not required for protection. .

## **Materials and methods**

### *Parasite and host*

Female wild-type (WT) C57BL/6 mice, and CD154<sup>-/-</sup> mice on a C57BL/6 background (25) were immunised with 500 irradiated (20-kilorad <sup>60</sup>Co source) *S. mansoni* cercariae via the abdomen (5, 10).

- 5 In some experiments, mice were treated with 100µg anti-CD40 mAb clone FGK45.5 (26), or control rat IgG (Sigma-Aldrich, Poole, UK), via the tail vein on days 1 and 3 p.v. and on days 6 and 10 (i.p). Alternatively, mice were treated with 1 µg murine rIL-12 (Genetics Institute, Cambridge, MA), or endotoxin-free 0.9% NaCl (Sigma-Aldrich), over the sternum (i.d.) on days 1 and 3 p.v. and on days 6 and 10 (i.p.). In selected experiments, 200 µl serum from naïve WT mice, or from vaccinated WT
- 10 mice (obtained 35 days p.v.) was administered i.v via the tail vein to vaccinated CD154<sup>-/-</sup> mice on day -1, and on days 1, 4 and 8 after exposure to challenge infection. All animal work was carried out in accordance with the guidelines of the United Kingdom Animals (Scientific Procedures) Act 1986.

### *Measurement of protection to challenge infection*

- 15 Vaccinated WT and CD154<sup>-/-</sup> mice, alongside naïve cohort mice (n = 5/group) were exposed to 200 cercariae via the tail 35 days p.v. Five weeks later, the adult worm burden in vaccinated (VC) and challenge control (CC) animals was enumerated following perfusion of the portal system. Resistance (R) to challenge infection was calculated (% R = [CC-VC/CC] x 100) in WT and CD154<sup>-/-</sup> mice

- 20 *Cell preparations and in vitro culture.*

Cell suspensions were prepared from the axillary LN that drain the abdomen and cultured as described previously (9). Broncho-alveolar lavage (BAL) cells were recovered from the lungs (27) and cultured at  $2.5 \times 10^5$  cells/well in 96-well flat bottomed plates (Nunc Surface, Nalge Nunc, Hereford, UK) in a total volume of 200µl, in the absence or presence of 40µg/ml soluble schistosomular antigen

- 25 preparation (SSAP) (9) for 48 hrs at 37°C / 5% CO<sub>2</sub>. Culture supernatants were removed and stored at -20°C until cytokine levels were assessed by ELISA.

### *Cytokine detection.*

Total RNA was extracted from lung tissue in TRIzol (Invitrogen, Paisley, UK) and amplified by

- 30 reverse transcription PCR (5). Genes analysed included; IL-12p40, IFNγ, IL-18, IL-23p19, IL-27p28, IL-4 and IL-5. PCR products were visualised by ethidium bromide and quantified by densitometry relative to GAPDH as a housekeeping gene. For detection of secreted cytokines, antibody ELISAs

were used to quantify IL-12p40, IFN $\gamma$ , IL-4 and IL-5 in the culture supernatants as described (8, 9). The lower limits of detection were 10 (IL-4), 40 (IL-12p40), and 50 (IFN $\gamma$ , IL-5) pg/ml. Nitric oxide (NO) production by cultured BAL cells was measured by Greiss assay as described (28).

#### 5 *Flow cytometric analysis of labelled cells*

BAL cells ( $1-2 \times 10^5$ ) were blocked with 4 $\mu$ l normal rabbit serum and then labelled with the following antibodies: FITC anti-CD11c (clone HL3; Pharmingen), biotin anti-CD4 (clone H129.19; Pharmingen) biotin anti-MHC II (I-A<sup>b,d</sup>, clone 28-16-8S; Caltag). Streptavidin-APC was used as a detection probe for biotin-conjugated antibodies. Irrelevant isotype-matched antibodies were used to determine levels of non-specific binding. Analysis was performed using a CyAn flow cytometer (DakoCytomation, UK).

#### *Tissue processing and histology of lung tissue*

Lung tissues were fixed in 10% neutral buffered formalin, embedded in wax, and then sectioned at 10 $\mu$ m. Tissue sections were stained with Harris' haematoxylin (BDH, Poole, UK), followed by 0.5% eosin (in 90% ethanol; BDH). At least 15 cellular foci were identified from each mouse.

#### *Antibody detection*

Serum obtained via tail bleeds at times post vaccination and challenge, were used to probe immunoplates (Maxisorb; Nunc) coated with 10 $\mu$ g / ml soluble worm antigen preparation (5). After washing with PBS-0.05% Tween20, bound antibodies were probed with goat anti-mouse IgG conjugated to horseradish peroxidase and revealed following the addition of SureBlue<sup>TM</sup> TMB substrate (Insight Biotechnology Wembly UK).

#### 25 *Statistics*

Comparisons of data were tested for significance with Student's *t* test. (\*\*\*,  $p < 0.001$ ; \*\*,  $p < 0.01$ ; \*,  $p < 0.05$ ; non-significant,  $p > 0.05$ ). Arithmetic means  $\pm$  the standard error of the mean (SEM) are shown. Data shown are representative of 2-4 experimental repeats.



## Results.

*CD40/CD154 interactions are essential for vaccine-induced protection.* Protective immunity to *S. mansoni* induced by the RA vaccine was determined in CD154<sup>-/-</sup> mice compared to WT cohorts. In WT mice, an average of 57% protection against challenge infection was induced (Fig.1,  $P < 0.001$  cf. challenge control mice), whereas vaccination of CD154<sup>-/-</sup> mice conferred no protection against challenge (-2% reduction in worm burden).

*Defects in Th1-associated cytokines but not cellular foci are evident in lungs of vaccinated CD154<sup>-/-</sup> mice.* As priming of the pulmonary tissues in the first 14-21 days after vaccination is a key event in the induction of protective immune responses (12), we investigated the lungs of CD154<sup>-/-</sup> mice at day 21 p.v.. Cellular foci that form around RA larvae after their arrival in the lungs of CD154<sup>-/-</sup> mice were similar in appearance but slightly smaller (average 17% reduction) than in WT mice (Fig. 2A). However, mRNA transcripts in the lungs CD154<sup>-/-</sup> mice showed a marked reduction in Th1-associated cytokines including IFN $\gamma$ , IL-12p40 and IL-27p28 (all  $P < 0.05$ ; Fig. 2B), although transcripts for IL-18 and IL-23p19 were not different in CD154<sup>-/-</sup> compared to WT mice ( $P > 0.05$ ). Th2-associated IL-4 and IL-5 transcripts were also not different between the two groups of mice (Fig. 2B).

*Administration of anti-CD40 mAb fails to restore Th1 responses in the lungs of vaccinated CD154<sup>-/-</sup> mice.* We previously showed that *in vivo* ligation of CD40 using an agonistic anti-CD40 mAb (to mimic the missing CD154) was sufficient to restore Th1 responses in the skin and sdLN of vaccinated CD154<sup>-/-</sup> mice (23). This was confirmed in the current study, as sdLN cells from CD154<sup>-/-</sup> mice given anti-CD40 mAb produced substantial amounts of IFN $\gamma$  at day 5, whereas cells from CD154<sup>-/-</sup> mice receiving control IgG did not (Fig. 3A). However, the effects of anti-CD40 mAb treatment (given on days 1, 3, 6 & 10 p.v.) were only transient since at day 15, sdLN cells from CD154<sup>-/-</sup> mice failed to produce IFN $\gamma$  when cultured with SSAP, regardless of prior antibody treatment (Fig. 3A). This contrasts with the sustained levels of SSAP-dependent IFN $\gamma$  production by WT sdLN cells at the same time-point. The failure to restore Th1-associated responses following anti CD40 mAb treatment was also seen in the lungs of mice 21 days p.v., as the production of spontaneous IL-12p40, plus antigen-driven IFN $\gamma$  was not significantly improved in treated compared to control CD154<sup>-/-</sup> mice (Figs. 3B and C). Although anti-CD40 mAb failed to restore Th1-associated cytokine production in the lungs of CD154<sup>-/-</sup> mice after vaccination, it was possible that a change would operate during the pulmonary

phase of challenge parasite migration. Significant levels of IL-12p40 were secreted by BAL cells from vaccinated CD154<sup>-/-</sup> recovered on day 15 p.c. but anti-CD40 mAb failed to elevate secretion of this cytokine (Fig 4D). Anti-CD40 mAb also failed to restore antigen-driven IFN $\gamma$  (Fig 4E).

5 *Administration of anti-CD40 mAb fails to restore protective immunity in CD154<sup>-/-</sup> mice.* An alternative effector immune response that may operate in RA vaccinated mice in addition to IFN $\gamma$ -mediated cellular responses, is the activity of anti-parasite antibodies (29, 30). The anti-worm IgG response in WT mice increased rapidly to the time of challenge infection, after which there was a progressive further increase (Fig. 4A). In CD154<sup>-/-</sup> mice however, no anti-worm IgG antibodies were observed at  
10 any time. Anti-CD40 mAb treatment of vaccinated WT mice did not significantly increase IgG production, nor did it restore antibody reactivity in CD154<sup>-/-</sup> mice.

Anti-CD40 mAb treatment also had no effect on the induction of protective immunity (Fig. 4B). Vaccinated WT mice harboured significantly lower worm burdens than challenge control mice whether  
15 they had received rat IgG or anti-CD40 mAb (68 and 66% respectively) but neither vaccinated CD154<sup>-/-</sup> mice given IgG, nor mice treated with anti-CD40 mAb, were significantly protected against challenge infection. (4.8% and -1.7% respectively).

*Exogenous IL-12 restores cellular immune responses in the lungs of CD154<sup>-/-</sup> mice.* To investigate  
20 why the absence of CD154 was lead to the failure to induce immune responses, even in the presence of a surrogate CD154 (*i.e.* anti-CD40 mAb), vaccinated CD154<sup>-/-</sup> mice were treated with rIL-12, as IL-12 production is one of the main functional consequences of CD40/CD154 signalling (17). We have previously shown that rIL-12 restores long-term Th1 responses in IL-12p40<sup>-/-</sup> mice, even when delivered only within the first few days after vaccination (5), and rIL-12 administered to CD154<sup>-/-</sup> mice  
25 completely restored IFN $\gamma$  production by sdLN cells on day 5 p.v. (23). Here we show that the inflammatory foci that formed in the lungs after challenge infection of CD154<sup>-/-</sup> mice were significantly smaller than those present in WT lungs (Fig. 5A-B). This contrasted with foci on day 21 p.v. when the foci were similar (*cf.* Fig 2A). Furthermore, treatment with rIL-12 restored foci size in CD154<sup>-/-</sup> mice back to WT levels (Fig. 5C and D). There was no significant difference in the number of foci present  
30 in the lungs of each group of mice (data not shown).

The cellular composition of the pulmonary immune response of rIL-12 treated-CD154<sup>-/-</sup> mice

recovered by BAL on day 21 p.v. and on day 15 p.c.(i.e. 12 and 40 days after final rIL-12 injection respectively) was markedly changed compared to CD154<sup>-/-</sup> mice treated only with saline. Based upon their size and granularity, the BAL cell population was divided into one of three cell groupings (Fig. 6A); small non-granular cells (lymphocytes), small granular cells (granulocytes), and large granular cells (macrophages and DCs). After vaccination (day 21 p.v.), the lungs of CD154<sup>-/-</sup> mice given saline had markedly decreased proportions of lymphocytes, which was verified by flow cytometric analysis of CD4<sup>+</sup> cells which were also significantly rarer than in WT mice (Fig 6B). The proportion of lymphocytes however, was restored following treatment with rIL-12 back to the level detected in WT mice. Treatment of CD154<sup>-/-</sup> mice with rIL-12 also substantially reduced the percentage of granulocytes (analysis by cytospin confirmed them to be eosinophils, data not shown) to less than that seen in the WT group. The numbers of CD11c<sup>+</sup> cells, which may be macrophages and or DCs (31), recovered by BAL on day 21 p.c.was also higher in CD154<sup>-/-</sup> mice given rIL-12 than the saline treated controls..

A similar changed pattern of BAL cell populations in CD154<sup>-/-</sup> mice treated with rIL-12 was detected after challenge infection (day 15 p.c.). Saline-treated CD154<sup>-/-</sup> lungs had a reduced proportion of lymphocytes compared to their WT counterparts ( $P < 0.05$ ), which was reflected by a reduced CD4<sup>+</sup> lymphocyte population ( $P < 0.05$ , Fig 6C). A large increase in the proportion of granulocytes was also detected from the lungs of CD154<sup>-/-</sup> mice given saline *cf.* WT mice ( $P < 0.05$ ; Fig 6C). However, treatment of CD154<sup>-/-</sup> mice with rIL-12 completely suppressed the increase in the proportion of granulocytes ( $P < 0.05$  *cf.* CD154<sup>-/-</sup> saline) and instead lead to a coincident restoration of the lymphocyte population in the lungs to a level comparable with WT mice.

Substantial differences in the level of activation (MHCII expression) of CD11c<sup>+</sup> cells were noted (Fig. 7). When compared to cells from naïve mice, the percentage of CD11c<sup>+</sup> cells from WT and CD154<sup>-/-</sup> mice given saline that expressed MHCII increased following vaccination and after challenge (Fig 7A-C). However, the expression of MHCII by cells from CD154<sup>-/-</sup> mice given saline was lower than their WT cohorts both after vaccination and after challenge. Remarkably, treatment of CD154<sup>-/-</sup> mice with rIL-12 significantly increased the proportion of MHC II<sup>+</sup> cells to over 90% ( $P < 0.01$  *cf.* CD154<sup>-/-</sup> saline), to the extent that it was not significantly different from WT mice ( $P > 0.05$  *cf.* WT).

*Exogenous IL-12 restores sustained pulmonary Th1 responses in CD154<sup>-/-</sup> mice and suppresses Th2 responses.* The BAL population of CD154<sup>-/-</sup> mice treated with saline were deficient in their production

of antigen specific IFN $\gamma$  and NO compared to WT mice at day 21 p.v. (Fig. 8A-B). However, in each case, administration of rIL-12 to CD154<sup>-/-</sup> mice, significantly restored the production of these mediators equivalent to, or above levels seen in vaccinated WT mice. BAL cells from saline-treated CD154<sup>-/-</sup> mice recovered after challenge also failed to produce IFN $\gamma$  and NO (Fig 8C and D). Importantly however, administration of rIL-12 given more than 5 weeks previously restored strong antigen-dependent IFN $\gamma$  and NO production by CD154<sup>-/-</sup> mice, equivalent to the levels produced by their WT counterparts (Fig. 8C and D). Compared to WT cells, CD154<sup>-/-</sup> lung cells produced significantly more IL-4 and IL-5 following stimulation with parasite antigen (both  $P < 0.01$ ; Fig. 8E and F), and as expected, the production of Th2 cytokines by CD154<sup>-/-</sup> mice was significantly inhibited by rIL-12 treatment.

*Exogenous IL-12 does not restore protection in CD154<sup>-/-</sup> mice.* Challenge parasite worm burdens were assessed in vaccinated CD154<sup>-/-</sup> mice treated with either saline or rIL-12, and compared to WT mice. Vaccination of WT mice induced 71% protection (Fig. 9). Unexpectedly, given their virtually identical pulmonary Th1 responses to WT mice, vaccinated CD154<sup>-/-</sup> mice given rIL-12 did not have significantly lower worm burdens than vaccinated saline-treated CD154<sup>-/-</sup> mice ( $P > 0.05$ ; 10.9% reduction).

*Serum from vaccinated WT mice does not restore protection in CD154<sup>-/-</sup> mice.* One major difference between vaccinated WT mice and vaccinated CD154<sup>-/-</sup> mice given rIL-12 was the magnitude of the anti-parasite antibody response. WT mice had substantial titres of anti-parasite IgG at day 15 p.c., whereas vaccinated CD154<sup>-/-</sup> mice (+/- rIL-12) did not (Fig. 10A). To assess whether the lack of IgG antibody production by CD154<sup>-/-</sup> mice was responsible for the lack of vaccine-induced protection, vaccinated CD154<sup>-/-</sup> mice were given four doses of vaccinated WT mouse serum (from one day prior to challenge up to day 8 p.c.). Control vaccinated CD154<sup>-/-</sup> mice received serum from naïve WT mice, or no serum. Treatment of CD154<sup>-/-</sup> mice with vaccine serum from WT mice restored significant titres of circulating parasite-specific IgG throughout the period of exposure to challenge parasites (Fig. 10B). Despite this, CD154<sup>-/-</sup> mice that had received vaccine serum were not significantly protected against challenge infection when compared to either untreated CD154<sup>-/-</sup> mice (Fig. 10C, 12 % reduction) or CD154<sup>-/-</sup> mice given naïve serum (16% reduction).

## Discussion.

Our studies show for the first time that CD40/CD154 signaling has an essential role in development of a protective immune response against schistosomes as CD154<sup>-/-</sup> mice exposed to the RA vaccine are not resistant to challenge infection. The complete absence of protective immunity in vaccinated CD154<sup>-/-</sup> mice is significant because the absence of many other immune components [e.g. IFN $\gamma$  (30), IFN $\gamma$ R (7), IL-4R $\alpha$  (32), IL-12p40 (5, 6) and IL-10 (6)], only leads to the partial abrogation of resistance. Our studies also reveal that the lack of protection is not simply due to the absence of IL-12-induced Th1-associated cellular immune responses in the lungs.

We previously demonstrated that CD154 is important for optimal expression of MHC II and CD86 by APCs in the skin; CD154 was also essential for CD4<sup>+</sup> cell proliferation in the sdLN, and for the production of Th1 but not Th2 cytokines (23). We now show that Th1-associated cytokine production in the lungs is also CD154 dependent, and the presence of IL-4 and IL-5 transcripts in the lungs of vaccinated CD154<sup>-/-</sup> mice combined with the secretion of IL-4 and IL-5 by BAL cells after challenge, reinforces our previous discovery that early Th2 cytokine production can be CD154 independent (23). In contrast to the sdLN, the lack of Th1 differentiation in the lungs of CD154<sup>-/-</sup> mice cannot be fully explained by the absence of CD40-dependent IL-12 production because CD154<sup>-/-</sup> lung cells produce measurable levels of IL-12p40 after challenge with normal cercariae. Furthermore, the absence of IFN $\gamma$  is not due to a lack of CD11c<sup>+</sup> APC or responder CD4<sup>+</sup> cells, as both are present (albeit in reduced numbers in the case of CD4<sup>+</sup> cells) in CD154<sup>-/-</sup> lungs. Instead, a more likely explanation is the immature phenotype of lung CD11c<sup>+</sup> cells in CD154<sup>-/-</sup> mice that express lower levels of MHC II. This may result from the relative lack of IL-12p40 and the absence of IFN $\gamma$ , both which feedback to further enhance APC maturation (33). Our studies also indicate a possible role for IL-27 in the promotion of Th1 differentiation (34) since in the absence of CD154, IL-27 transcripts in the lungs were markedly reduced. BAL cells from the lungs of vaccinated CD154<sup>-/-</sup> mice not only lack optimum expression of MHC II, they also do not express NO. This is consistent with other studies showing that macrophages produce NO via a CD40/CD154 dependent mechanism (35-37). Although NO may have only a limited direct role in protection against schistosomes (28, 38), its lack of production is further evidence of poor macrophage activation in CD154<sup>-/-</sup> mice.

The restoration of Th1-associated immune responses in the sdLN of CD154<sup>-/-</sup> mice by the administration of anti-CD40 mAb (23), raised the possibility that this treatment may induce protective

immunity in vaccinated CD154<sup>-/-</sup> mice. Indeed, anti-CD40 mAb treatment of WT mice can induce strong Th1 responses in a variety of experimental models through both IL-12 dependent and independent pathways (20, 24, 39, 40). However, it is possible the defective cellular response and the absence of protection in vaccinated CD154<sup>-/-</sup> mice was either the result of a failure of signalling through CD40 into the APC (responsible for APC maturation and IL-12 production), or a lack of signalling through CD154 into the CD4<sup>+</sup> cell (responsible for CD4<sup>+</sup> cell activation). Consistent with previous studies (23), anti-CD40 mAb treatment of vaccinated CD154<sup>-/-</sup> mice restored antigen-induced IFN $\gamma$  by sdLN cells at day 5 showing that signals transduced through CD154 directly into CD4<sup>+</sup> cells are not required for initial Th1 differentiation. This agrees with other studies showing that Th1 responses can be restored in CD154<sup>-/-</sup> mice through anti-CD40 mAb treatment (41). However, the effect of anti-CD40 mAb treatment in the present studies was only transient and the restorative effect on IFN $\gamma$  production in the sdLN was not detected by day 15. In addition, no increase was detected in the production of IL-12p40, IFN $\gamma$ , or NO by BAL cells p.v., or p.c. following treatment with anti-CD40 mAb. Treatment of vaccinated CD154<sup>-/-</sup> mice also did not restore anti-parasite antibody production, in agreement with a study on allograft immunity (42), although other studies demonstrate that anti-CD40 mAb can restore antibody isotype class switching (41, 43).

In the context of the all results described above, it was perhaps not surprising that anti-CD40 mAb failed to restore protection in CD154<sup>-/-</sup> mice; it also failed to boost immunity in WT mice. Nevertheless, the result was still disappointing because similar treatment regimes lead to sustained protective effects in other disease models (39, 40, 44, 45). The transient nature of the response to anti-CD40 mAb is unlikely to be caused by immune-mediated clearance, as CD154<sup>-/-</sup> mice do not produce detectable IgG antibody titres against the anti-CD40 mAb molecule itself (data not shown). It is also unlikely that the treatment causes the death / inactivation of CD40<sup>+</sup> cells (46), since anti-CD40 mAb treatment does not impede vaccine-induced immune responses in WT mice. One interpretation is that CD40 stimulation alone, whilst sufficient to induce short-term Th1 differentiation, cannot support the development of long-term Th1 clones and that CD154 is required for the generation of memory Th1 clones after vaccination (47).

An important consequence of CD40/CD154 interaction is CD40-dependent IL-12 production which we showed is defective in vaccinated CD154<sup>-/-</sup> mice. In order to circumvent this deficiency, rIL-12 was administered to CD154<sup>-/-</sup> mice. Administration of rIL-12 stimulated elevated levels of antigen-specific

IFN $\gamma$  production by sILN cells in CD154<sup>-/-</sup> mice (23), consistent with its known ability to promote Th1 differentiation in the RA vaccine model (5, 48). The effects of rIL-12 are long lasting, as vaccinated CD154<sup>-/-</sup> mice made strong pulmonary Th1 responses both after vaccination and after challenge (12 and 40 days respectively after cessation of rIL-12 treatment). This indicates that the generation of memory Th1 cells does not require signalling through CD154, and implies that the main functional consequence of CD40/CD154 interaction is the production of IL-12 which supports Th1 memory cell development. In support of our conclusion, treatment of mice with rIL-12 reverses the inhibitory effects of CD40/CD154 blockade on intestinal inflammation (49) and protection to *L. major* (21). The long-term effects of rIL-12 treatment may be due to its ability to stimulate *de novo* autocrine IL-12, and IFN $\gamma$  in the continued absence of CD40/CD154 interactions. Given the short half life of rIL-12 *in vivo* (~3 hours), it is unlikely that the IL-12p40 detected in BAL culture supernatants is the injected rIL-12 molecule. Since one of the main consequence of CD40 ligation is the release of IL-12p40, it is surprising that rIL-12 is a much more potent stimulus of Th1 responses than anti-CD40 mAb. The differential effects can be most likely explained in terms of the amount of rIL-12 used, approximating to the quantities used in other studies (5, 48, 50), compared to treatment of mice with anti-CD40 mAb induced the production of only up to 2 ng IL-12p40 by skin cells (23).

The potent and prolonged effects of rIL-12 are illustrated by its influence on the cell types present in the lungs after vaccination and challenge. For example, the proportion of CD4<sup>+</sup> cells in the lungs of vaccinated CD154<sup>-/-</sup> mice both p.v. and p.c. was reduced but was restored to WT levels by rIL-12 treatment. This implies that rIL-12 increases either the efficiency of recruitment, or delays the death, of CD4<sup>+</sup> cells in the lungs. The lungs of CD154<sup>-/-</sup> mice also contain an enhanced proportion of small granular cells which are thought to be eosinophils. Substantial pulmonary eosinophilia develops in the lungs of vaccinated IFN $\gamma$ R<sup>-/-</sup> (7) and IL-12p40<sup>-/-</sup> mice (5) that fail to produce Th1 cytokines but instead mount elevated parasite-specific Th2 responses. BAL cells from vaccinated CD154<sup>-/-</sup> mice also produce elevated levels of IL-4 and IL-5 after challenge contradicting several studies which show that CD40/CD154 interactions are essential for the Th2 development caused by helminth infection (51-54) but supporting others (55). Nevertheless, it is interesting to note that protective immunity was absent in vaccinated CD154<sup>-/-</sup> mice which exhibit exaggerated Th2 cytokine production in their lung responses after challenge, whereas a Th2 bias has previously been correlated with a significant although limited degree of protection (5, 7). The Th2 cytokine bias however, was not apparent in the first few weeks after vaccination, and was only noted after challenge perhaps because the absence of IL-12 and IFN $\gamma$  in

vaccinated CD154<sup>-/-</sup> mice allows the eventual development of Th2 responses after a few weeks by default.

Despite the apparent complete restoration of the pulmonary immune response akin to that in protected WT mice, treatment of CD154<sup>-/-</sup> mice with rIL-12 does not restore protection against subsequent challenge. This was surprising since rIL-12 restored high levels of protection against schistosomes in IL-12p40<sup>-/-</sup> mice (5), and treatment of CD154<sup>-/-</sup> mice with rIL-12 restored immunity against *L. major* (21). Thus, protection induced by the RA schistosome vaccine is completely dependent on the CD40/CD154 pathway but not upon the presence of IL-12-driven Th1-type immune responses. An IL-12-independent role for CD40/CD154 interactions is also evident in the development of crescentic glomerulonephritis caused by the presence of pathogenic Th1 cells in the kidneys (56). In this example, treatment with rIL-12 restores Th1 responses but still fails to induce pathology, coincident with a lack of activation of kidney macrophages. Our studies and those of Ruth et al. (56), demonstrate that rIL-12 alone cannot compensate for all immune deficiencies that result from the disruption of the CD40/CD154 pathway. Our results also show that CD154<sup>-/-</sup> mice given rIL-12 are another example, in addition to IL-4Rα<sup>-/-</sup> and TNFR<sup>-/-</sup> mice (32, 57) of hosts that generate normal pulmonary foci to challenge infection in schistosome vaccinated mice but exhibit little or no protection.

One example of an IL-12-independent, CD40/CD154-dependent, immune response is the class switch in antibody production to IgG isotypes. Antibodies appear to have a limited role in the protective immune response induced by RA schistosomes (29, 30, 58), although the precise mechanism is not clear since the expression of FcγR is not required (30). Vaccinated CD154<sup>-/-</sup> mice (+/- rIL-12) do not produce anti-parasite IgG antibodies, consistent with a crucial role for CD40/CD154 interactions in antibody class switching (51, 56). However, although CD154<sup>-/-</sup> mice given serum from vaccinated WT mice had significant levels of circulating anti-parasite IgG antibodies, this treatment did not restore any level of protection. Transfer of resistance into naïve recipients with serum from WT mice exposed to a single dose of RA larvae is generally unsuccessful (59), but the transfer of such serum into vaccinated B cell deficient (μMT) mice can increase protection from ~40% to upwards of 70% (30), whilst serum transfer into naïve IL-4Rα mice also confers a significant reduction in the challenge worm burden (32). These reports show that in principle delivery of vaccine serum from WT mice into transgenic mice with a deficiency in antibody production can transfer some degree of protection, but underline the present



study's inability to confer even a limited level of protection in vaccinated CD154<sup>-/-</sup> mice by passive transfer of vaccinated serum.

In conclusion, the failure of immune serum to transfer protection to vaccinated CD154<sup>-/-</sup> mice suggests that protection to the RA vaccine is not solely dependent upon the presence of anti-parasite antibodies. Instead, although CD154 is essential for the switching to IgG antibody production, which may have an anti-parasite effect, some other CD154-dependent effector mechanism is responsible for challenge parasite elimination. In turn, this mechanism does not appear to involve inflammatory cell reactions in the lungs that can be induced following the administration of IL-12. The effector mechanism may rely upon the interaction of coincident IgG humoral responses with an as yet unidentified CD154-mediated cellular reaction.

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### Legends to Figures.

Figure 1.

*CD154 is essential for protective immunity.* Worm burdens of challenge control (open bars) and vaccinated and challenged (solid bars) mice 5 weeks after infection with 200 normal cercariae. Bars show the mean + SEM for each group. The percentage value is the calculated level of resistance in vaccinated mice compared to challenge control mice within each group. Significance values refer to the difference between worm burdens in vaccinated compared with the relevant challenge control group.

Figure 2.

*Immune responses in the lungs of CD154<sup>-/-</sup> mice after vaccination.* **A.** Representative inflammatory foci in the lungs of vaccinated WT and CD154<sup>-/-</sup> mice on day 21 p.v.. Tissue sections stained with H&E; scale bars are 50 µm. **B.** Gene transcripts in lung tissue on day 21 p.v. for 3 individual WT and CD154<sup>-/-</sup> mice. Significance values relate to the mean densitometry value for each cytokine transcript normalized against the house keeping gene GAPDH.

Figure 3.

*Administration of anti-CD40 mAb does not restore long-term Th1-type responses in vaccinated CD154<sup>-/-</sup> mice.* **A.** Production of IFNγ by antigen-stimulated sdLN cells from WT and CD154<sup>-/-</sup> mice on days 5 and 15 p.v. **B & D,** production of spontaneous IL-12p40 by unstimulated BAL cells, and **C & E,** production of IFNγ by antigen stimulated BAL cells, on day 21 p.v. (**B & C**), and day 15 p.c. (**D & E**). Values are mean + SEM for groups of mice (n = 5 mice / group) given control rat IgG (open bars) or anti-CD40 mAb (solid bars). Horizontal dashed lines denote minimum detection level for IL-12p40. Significance values are for anti-CD40 mAb-treated mice compared to control IgG treated mice.

Figure 4.

*Administration of anti CD40 mAb does not restore antibody production, or protective immunity in CD154<sup>-/-</sup> mice.* **A.** Level of anti-parasite IgG levels in WT (triangles) and CD154<sup>-/-</sup> mice (circles) given control rat IgG (solid line, filled symbols), or anti-CD40 mAb (dashed line, open symbols). Anti-CD40 mAb, or control IgG were administered on 1, 3, 6 and 10 p.v. Small arrows indicate administration of anti-CD40 mAb, single large arrow denotes the time of challenge infection. **B.** Worm burdens of challenge control (open bars) and vaccinated and challenged (solid bars) mice 5 weeks p.c. Bars are the mean + SEM in each group. The percentage resistance and significance values refer to mean worm burdens in vaccinated / challenged compared to challenge control mice.

Figure 5.

*IL-12 restores the development of inflammatory foci in the lungs of CD154<sup>-/-</sup> mice after challenge.*

Representative inflammatory foci in the lungs of vaccinated and challenged **A.** WT mice, **B.** CD154<sup>-/-</sup> + saline mice, and **C.** CD154<sup>-/-</sup> + rIL-12 mice, 15 days p.c.. Tissue sections stained with H&E; scale bars are 200 µm. Diameter in µm (mean + SEM) of pulmonary foci (n =15-20) at day 15 p.c; significance values are for CD154<sup>-/-</sup> + saline mice connected to either WT mice, or CD154<sup>-/-</sup> + rIL-12 mice.

Figure 6.

*IL-12 restores the BAL cell composition after vaccination and after challenge.* **A.** Representative scatter plots of BAL cells recovered from the lungs of WT (left) and CD154<sup>-/-</sup> mice (right) showing size (forward scatter) and granularity (side scatter). Regions are: L = lymphocytes, G = granulocytes, LG = large granular macophages and DCs. **B.** Proportions of BAL cell types in WT mice, CD154<sup>-/-</sup> mice given saline, and CD154<sup>-/-</sup> mice given rIL-12, on day 21 p.v., and **C.** on day 15 p.c.. IL-12 was given on days 1, 3, 6 and 10 p.v.. Small non-granular lymphocytes (open), granulocytes (light grey) and large granular macrophages and DCs (dark grey) cells as determined by flow cytometric analysis, shown as histogram bars + SEM (n = 4

mice / group). Proportions of CD4<sup>+</sup> and CD11c<sup>+</sup> cells in BAL labeled with specific antibodies and analysed by flow cytometry shown as bars + SEM. Significance values are for CD154<sup>-/-</sup> mice given saline compared to WT mice, or CD154<sup>-/-</sup> mice given rIL-12.

5 Figure 7.

*Expression of MHCII on CD11c<sup>+</sup> BAL cells is restored by administration of rIL-12.* Flow cytometric analysis of MHCII<sup>+</sup> cells *versus* autofluorescence of CD11c<sup>+</sup> BAL cells recovered on; **A.** day 0 from WT and CD154<sup>-/-</sup> mice, **B.** day 21 p.v. from WT mice, CD154<sup>-/-</sup> + saline mice, and CD154<sup>-/-</sup> + rIL-12 mice, and **C.** day 15 p.c. from the same 3 groups. Plots are a representative example of 4 mice in each group. Percent values given are the mean value of MHC II<sup>+</sup> cells in region R1 determined relative to staining with an isotype control.

Figure 8.

*Th1-associated cytokines are restored and Th2 cytokines are ablated in CD154<sup>-/-</sup> mice treated with rIL-12*

15 Production of **(A)** spontaneous IL-12p40 by unstimulated BAL cells, and production of **(B and D)** IFN $\gamma$ , **(C and E)** NO, **(F)** IL-4, and **(G)** IL-5, by SSAP-stimulated BAL cells recovered from WT mice, CD154<sup>-/-</sup> + saline mice, and CD154<sup>-/-</sup> + rIL-12 mice, on day 21 p.v. **(A-C)**, or day 15 p.c. **(D-G)**. Values are mean + SEM for groups of mice (n=3-4 mice / group); significance values are for CD154<sup>-/-</sup> + saline mice connected to either WT mice, or CD154<sup>-/-</sup> + rIL-12 mice. Horizontal dotted lines denote minimum detection levels.

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Figure 9.

*IL-12 does not restore protective immunity in CD154<sup>-/-</sup> mice.* Worm burdens of challenge control (open bar) and vaccinated and challenged (light grey hatched bar) WT mice 5 weeks after challenge infection with 200 normal cercariae. Worm burdens in vaccinated and challenged CD154<sup>-/-</sup> mice given saline (dark grey hatched bar) and CD154<sup>-/-</sup> mice treated with rIL-12 (solid bar). Bars are the mean + SEM in each group.

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Figure 10.

*Passive transfer of vaccinated WT mouse serum does not confer protection to CD154<sup>-/-</sup> mice.* **A.** Anti-parasite IgG levels in WT mice (triangles, solid line), CD154<sup>-/-</sup> mice given saline (solid circle, solid line) and CD154<sup>-/-</sup> mice treated with rIL-12 (open circle, dotted line).at day 15 p.c. **B.** Levels of anti-parasite IgG in vaccinated CD154<sup>-/-</sup> mice that had received serum from vaccinated WT mice (injections indicated by arrows on days -1, 1, 4, and 8 p.c.), measured at intervals after challenge infection (inverted triangle, solid line). Antibody levels in CD154<sup>-/-</sup> mice recipients of no serum (solid circle, solid line), and recipients of naïve WT serum (open squares, dashed line). Floating black line is the level anti-parasite IgG in vaccinated serum from WT mice prior to administration; the floating dashed bar is the level of anti-parasite antibody in naïve serum. **C.** Worm burdens of vaccinated and challenged untreated CD154<sup>-/-</sup> mice (open bar), CD154<sup>-/-</sup> recipients of naïve serum (grey bar), and CD154<sup>-/-</sup> recipients of vaccinated serum from WT mice (black bar). Worm burdens determined 5 weeks after challenge with 200 normal cercariae; bars are the mean + SEM in each group (n = 5 mice).

Figure 1.

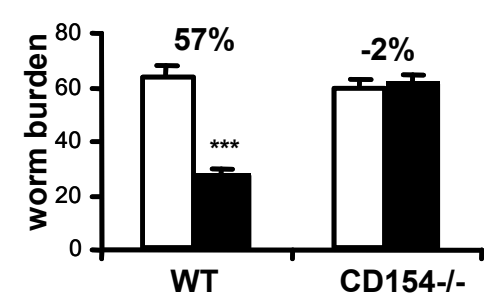


Figure 2.

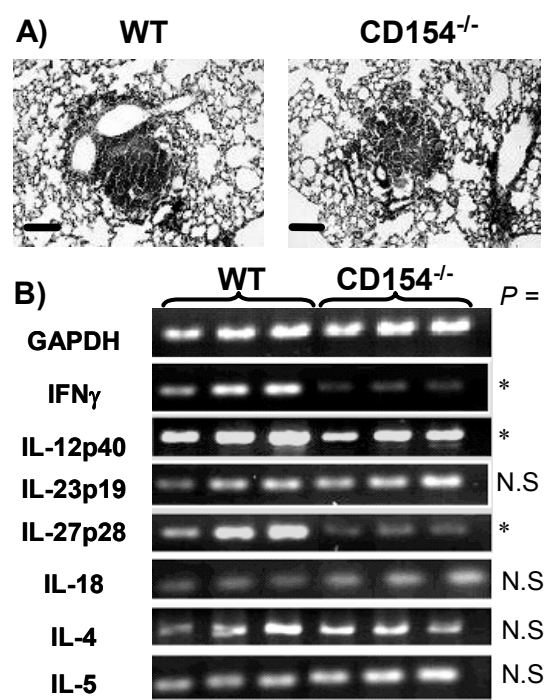
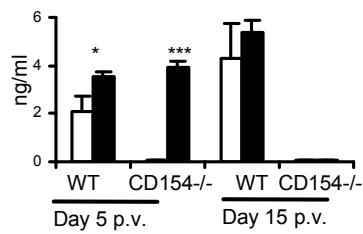
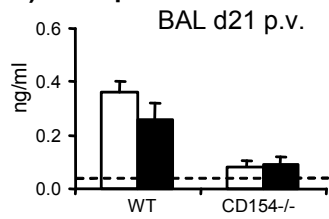


Figure 3.

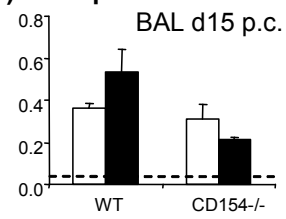
**A) IFN $\gamma$ :** sdLN – d5 & d15 p.v.



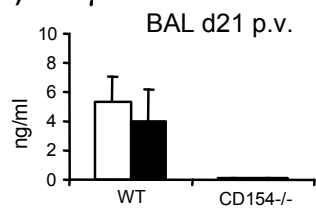
**B) IL-12p40:**



**D) IL-12p40:**



**C) IFN $\gamma$ :**



**E) IFN $\gamma$ :**

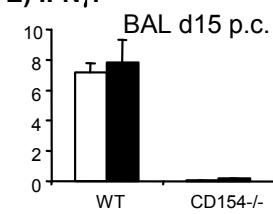
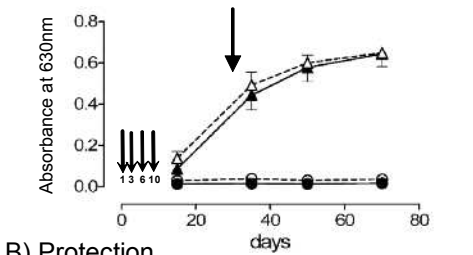


Figure 4

A) Antibody titre



B) Protection

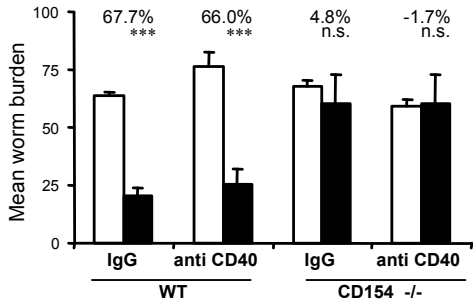


Figure 5

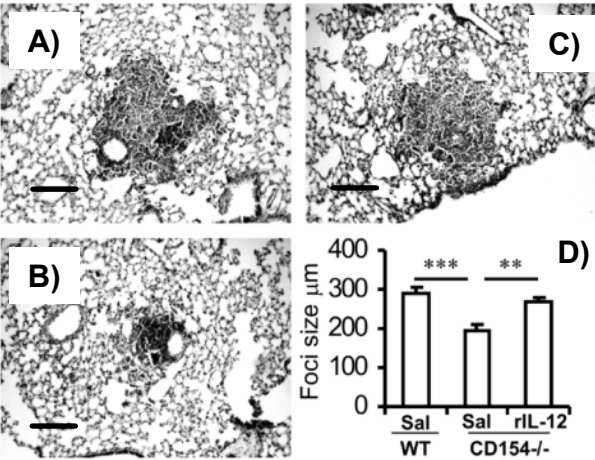


Figure 6

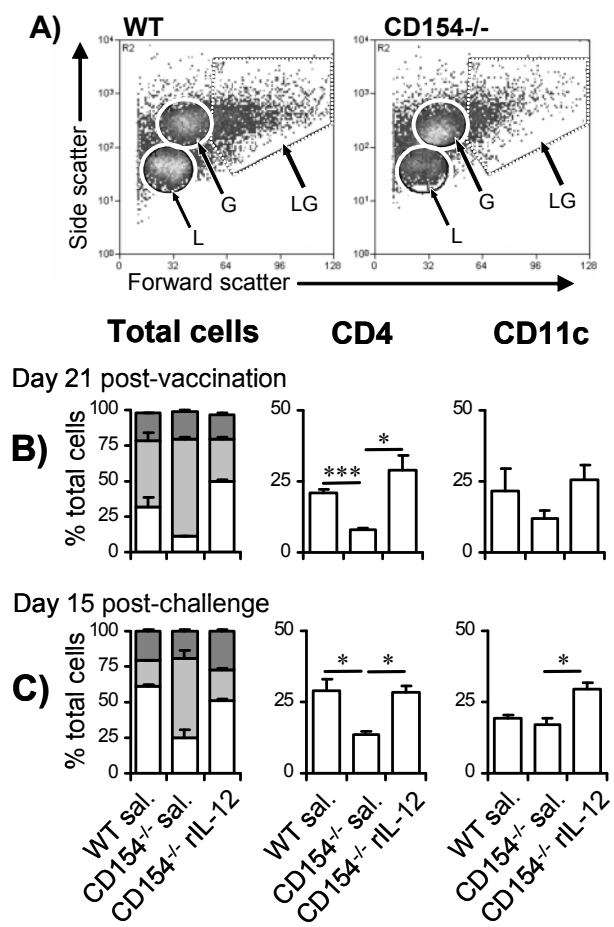


Figure 7.

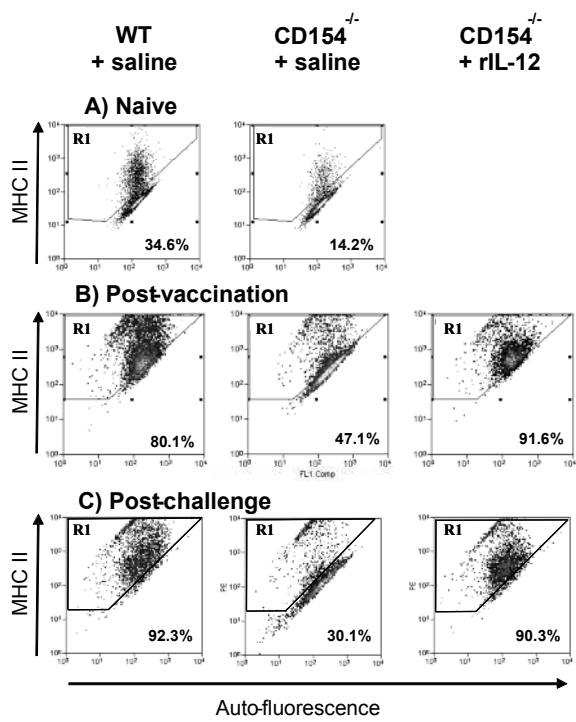




Figure 8

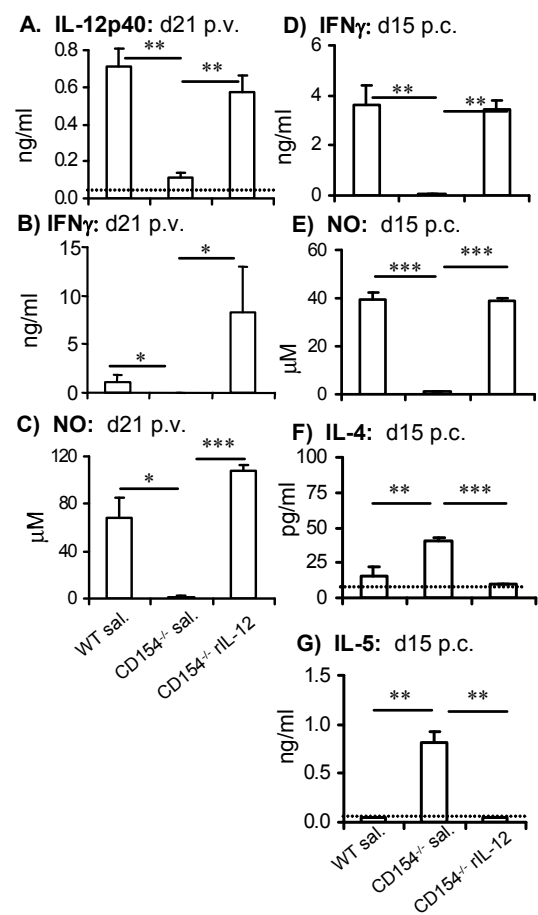


Figure 9

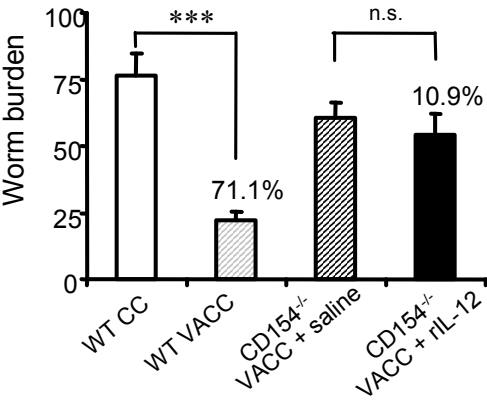


Figure 10

